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FtsY, the bacterial signal-recognition particle receptor, interacts functionally and physically with the SecYEG translocon

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Co-translational membrane targeting of proteins by the bacterial signal-recognition particle (SRP) requires the specific interaction of the SRP–ribosome nascent chain complex with FtsY, the bacterial SRP receptor (SR). FtsY is homologous to the SRα-subunit of the eukaryotic SR, which is tethered to the endoplasmic-reticulum membrane by its interaction with the integral SRβ-subunit. In contrast to SRα, FtsY is partly membrane associated and partly located in the cytosol. However, the mechanisms by which FtsY associates with the membrane are unclear. No gene encoding an SRβ homologue has been found in bacterial genomes, and the presence of an FtsY-specific membrane receptor has not been shown so far. We now provide evidence for the direct interaction between FtsY and the SecY translocon. This interaction offers an explanation of how the bacterial SRP cycle is regulated in response to available translocation channels.

Keywords: SRP; FtsY; SRP receptor; SecYEG; co-translational targeting

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INTRODUCTION

Membrane-protein assembly in bacteria is initiated by the co-translational interaction of the bacterial signal-recognition particle (SRP) with the signal anchor sequence of a newly synthesized membrane protein. Subsequently, the SRP–ribosome nascent chain (SRP–RNC) complex is targeted to the membrane by its interaction with FtsY, the bacterial homologue of the α-subunit of the eukaryotic SRP receptor (SR; Koch *et al*, 2003; Wild *et al*, 2004). In mammalian cells, SRα is anchored by its A-domain to the integral membrane subunit SRβ (Young *et al*, 1995). SRβ not only serves as a membrane anchor for SRα, but is also thought to be involved in coordinating signal sequence release from SRP54, with RNC binding to the translocon (Song *et al*, 2000; Fulga *et al*, 2001; Schwartz & Blobel, 2003). In contrast to SRα, FtsY is located partly in the cytosol and partly at the cytoplasmic membrane (Luirink *et al*, 1994). However, the mechanisms by which FtsY is bound to the membrane are still unclear. No homologue of SRβ has been identified in bacterial genomes, and most bacterial FtsY homologues do not contain a transmembrane segment (Bibi *et al*, 2001). Recent studies have suggested the involvement of phosphatidylethanolamine (PE) in the membrane association of FtsY (de Leeuw *et al*, 2000; Millman *et al*, 2001). However, PE accounts for more than 70% of the membrane phospholipids, and it seems unlikely that the binding of FtsY to an abundant lipid is sufficient to ensure specific targeting of an SRP–RNC complex to the limited number of SecYEG translocons in the membrane. It has been proposed recently that membrane assembly of FtsY occurs in a two-step process involving an initial binding to PE and subsequent transfer to a membrane-bound receptor (Millman *et al*, 2001). We now present evidence that FtsY is able to interact directly with the Sec translocon, which could indicate that the Sec translocon functions as the membrane-bound receptor for FtsY.

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RESULTS AND DISCUSSION

The *secY40* mutation blocks membrane-protein integration

Extensive mutagenesis studies on SecY, the central component of the SecYEG translocon, have indicated an essential role of cytoplasmic loops C5 and C6 in the translocation of SecA-dependent proteins (Mori & Ito, 2001). Although most of the available *secY* mutants have not been screened for their effect on membrane-protein biogenesis, *in vivo* studies have shown that membrane-protein integration is severely reduced in the cold-sensitive *secY40* mutant (Newitt & Bernstein, 1998). This mutant carries an A^{363}-T exchange within the conserved S^{349}-Y^{365} segment of the cytoplasmic loop C5 (Taura *et al*, 1994), suggesting that this loop is also involved in the integration of SRP-dependent membrane proteins.

To characterize the molecular basis of the *secY40* defect, we performed an *in vitro* study using inner membrane vesicles (INV) derived from the *secY40* strain. In agreement with previous *in vivo* data (Newitt & Bernstein, 1998), the translocation of SecA-dependent proteins, such as OmpA, was not affected by the *secY40* mutation (Fig 1). Conversely, the integration of mannitol permease (MtlA; the SRP-dependent and SecA-independent

polytopic membrane protein; Koch *et al*, 1999) was severely reduced in *secY40* INV (Fig 1). The same integration defect was observed for the polytopic membrane protein YidC (Fig 1), which—like MtlA—is targeted to the SecYEG translocon by SRP, but requires SecA in addition for the translocation of a large periplasmic loop (Koch *et al*, 2002).

The *secY40* mutation did not have an effect on the integration of the M13 coat protein (Fig 1A), which integrates independently of SecYEG by a novel YidC-dependent integration pathway (Samuelson *et al*, 2000). This suggests that the integration defects of MtlA and YidC are not the result of indirect effects induced by the *secY40* mutation, such as reduced YidC levels in the membrane.

FtsY is able to suppress the *secY40* phenotype

In the recently solved crystal structure of the SecY translocon (van den Berg *et al*, 2004), the cytoplasmic loop C5 is surface exposed, which would allow interaction with cytosolic factors during protein transport. The observation that the *secY40* mutation specifically blocks the integration of SRP-dependent membrane proteins prompted us to analyse whether increasing concentrations of Ffh (the protein component of bacterial SRP) or of FtsY would restore integration into the *secY40* INV *in vitro*. Strikingly, the addition of purified FtsY restored integration of MtlA into *secY40* INV to near wild-type (wt) level (Fig 2A, lanes 6, 10). Conversely, the integration of MtlA into wt INV was not amplified by increasing the FtsY concentration (Fig 2A, compare lane 4 with lane 8), suggesting that the FtsY concentration in our *in vitro* system was not limiting for integration. In contrast, the addition of Ffh to *secY40* INV did not stimulate MtlA integration (Fig 2A, compare lane 12 with lane 14). This was not due to limiting concentrations of 4.5S RNA, which, together with Ffh, forms the bacterial SRP, because the *in vitro* system contains sufficient amounts of 4.5S RNA (Koch *et al*, 1999) and the simultaneous addition of Ffh and 4.5S RNA did not improve MtlA integration into *secY40* INV (data not shown).

To determine the specificity of SecY mutation suppression by FtsY, we also tested INV derived from the *secY39* mutant, which carries an R^{357}-H exchange only six residues away from that mutated in the *secY40* mutant. The *secY39* mutant blocks the translocation of SecA-dependent secretory proteins as well as the integration of SRP-dependent membrane proteins (Koch & Müller, 2000; Mori & Ito, 2001). FtsY was unable to restore the integration defect of *sec39* INV (Fig 2A, lanes 15–18), suggesting that Ala^{363}, which is mutated in the *secY40* mutant, has a specific role in the SecY–FtsY interaction during co-translational integration.

Much like MtlA, the membrane protein YidC could not be efficiently integrated into the *secY40* INV unless additional FtsY was added (Fig 2B). The specificity of the *secY40* suppression by FtsY was further analysed using FtsY-307, a truncated derivative of FtsY. FtsY-307 consists of the amino-terminal domain of FtsY, which is sufficient for membrane binding (de Leeuw *et al*, 1997; Millman *et al*, 2001), but lacks the GTPase domain. Purified FtsY307 was unable to promote the integration of YidC into the *secY40* INV (Fig 2B) at concentrations sufficient for suppression by full-size FtsY. FtsY-307 was also unable to support integration of MtlA into the *secY40* INV (data not shown), suggesting that this suppression probably requires the GTPase domain of FtsY.

We next tested whether FtsY was able to suppress the *secY40* cold-sensitive phenotype *in vivo*. We introduced the T7-RNA polymerase gene into the *secY40* strain by transduction and subsequently transformed the cells with pET19b-FtsY—a plasmid expressing FtsY under the control of the T7 promoter. At permissive conditions (37 °C), we did not observe a significant difference in the growth rate between wt and *secY40* cells (Fig 2C, right panel). If the cells were grown at 25 °C, however, a significant growth defect was observed for the *secY40* strain. Strikingly, the growth defect was significantly reduced by moderate FtsY expression from plasmid pET19b-FtsY (Fig 2C, left panel). Our attempts to suppress completely the cold-sensitive phenotype by increasing FtsY expression failed, because high FtsY levels inhibited growth in both *secY40* and wt cells even at 37 °C

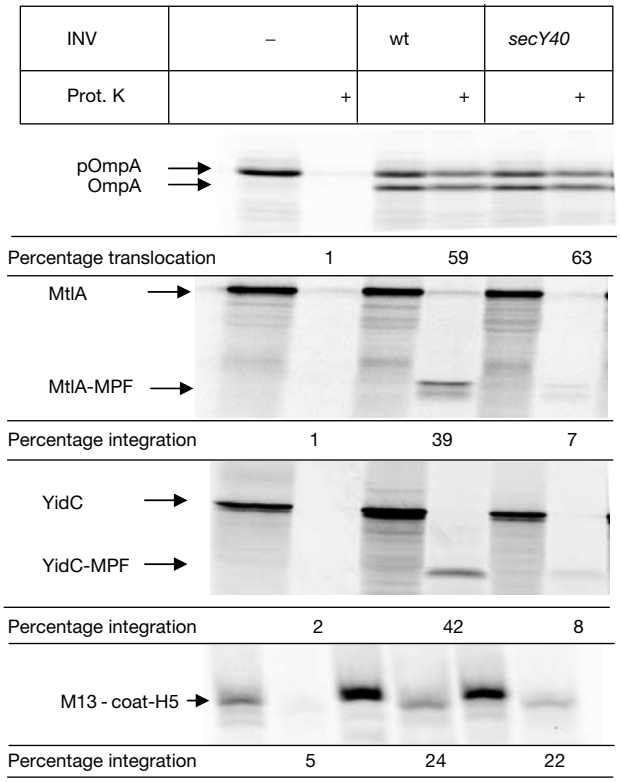

Fig 1 | The *secY40* mutation blocks the integration of SRP-dependent membrane proteins. pOmpA, MtlA, YidC and M13-coat-H5 were synthesized *in vitro* at 25 °C for 45 min in the absence or presence of inside-out inner membrane vesicles derived from either wild-type *Escherichia coli* cells or the *secY40* mutant strain. Translation products were precipitated either directly with trichloroacetic acid (TCA) or after incubation with 0.5 mg/ml proteinase K (Prot. K) for 20 min at 25 °C. The positions of the precursor (pOmpA) and the mature form of OmpA are indicated, along with the positions of full-size MtlA and YidC and their proteinase K-resistant membrane-protected fragments (MPF). M13-coat-H5, an M13 coat derivative lacking the signal sequence cleavage site (Kuhn & Wickner, 1985), was separated on 22% urea/SDS–PAGE, and all other samples were separated on 13% SDS–PAGE. The percentage of translocation or integration was calculated after quantification of the radioactivity of the individual protein bands using a PhosphorImager and ImageQuant software, and by calculating the ratio between the amounts present in the proteinase K-treated sample and the TCA-precipitated sample.

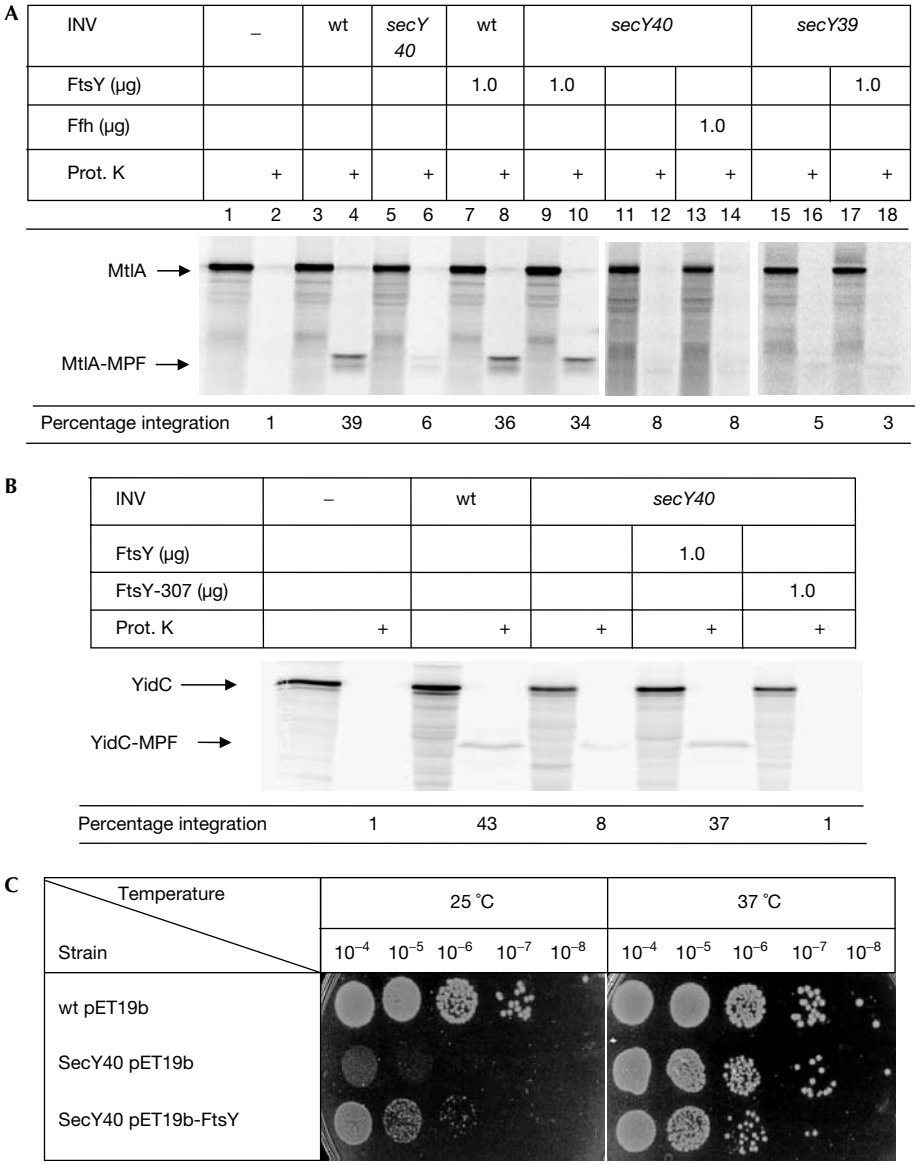

Fig 2 | FtsY suppresses the *secY40* phenotype both *in vivo* and *in vitro*. (**A**) MtlA was synthesized *in vitro* in the presence of wild-type (wt), *secY40* or *secY39* INV as shown in Fig 1. When indicated, purified FtsY or Ffh was added. Prot. K, proteinase K. (**B**) YidC was synthesized *in vitro* in the presence of wt or *secY40* INV. Purified FtsY or FtsY-307 (a truncated FtsY derivative lacking the GTPase domain) was added when indicated. (**C**) *SecY40* cells expressing FtsY were incubated in liquid Luria–Bertani medium at 37 °C. After overnight culture, the culture was serially diluted and spotted on LB plates containing 0.2 mM isopropyl β-D-thiogalactopyranoside. Plates were incubated at either 37 or 25 °C. *SecY40* and wt cells carrying the empty vector were used as controls.

(data not shown). In summary, our data show that the *secY40* mutation can be suppressed both *in vivo* and *in vitro* by increasing the concentration of the bacterial SRP receptor FtsY.

In vitro studies show an FtsY–SecY interaction

The data presented so far suggest a direct interaction between SecY and FtsY. We had shown previously that *in vitro*-synthesized and integrated SecY assembles into a biologically active translocon complex (Koch & Müller, 2000). This allowed us to analyse the interaction between *in vitro*-synthesized SecY and FtsY by chemical crosslinking. ^{35}S-labelled SecY was first synthesized *in vitro* and integrated into INV before the INV were treated with urea to remove any SecY molecules that were not fully integrated and also the endogenous, INV-bound FtsY. Subsequently, these INV were incubated with the soluble crosslinker BS^3 in the presence of an FtsY-containing or FtsY-depleted cytosolic extract. In the presence of FtsY, a strong radiolabelled crosslink of about 140 kDa was observed, which was not present in the absence of FtsY (Fig 3). This crosslinking product was immunoprecipitated with both α-FtsY and α-SecY antibodies, indicating the presence of a SecY–FtsY complex. A second crosslinking product of about 110 kDa, visible only in the presence of FtsY, was also

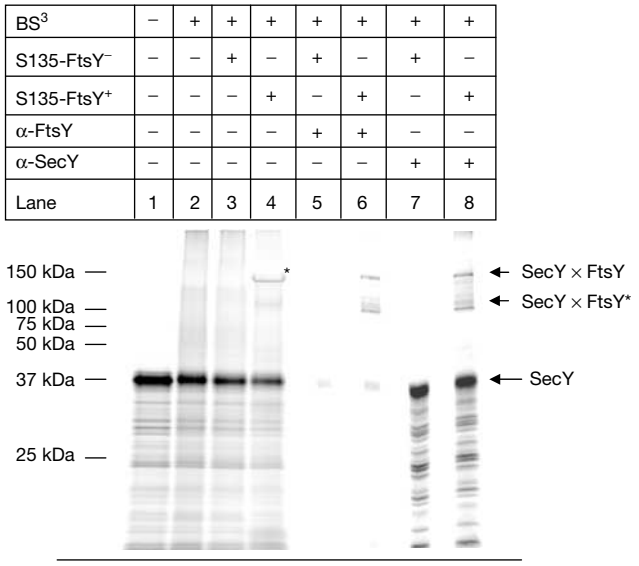

Fig 3 | Chemical crosslinking shows the interaction between FtsY and SecY. INV containing *in vitro*-synthesized SecY were treated with urea to remove membrane-bound FtsY and were subsequently incubated with either buffer or cytosolic extracts derived from cells depleted of FtsY (S135-FtsY$^-$) or from cells expressing FtsY (S135-FtsY$^+$). Crosslinking was performed with the soluble crosslinker BS^3. Crosslinked bands were identified in a sixfold scaled-up reaction mixture by immunoprecipitation using α-FtsY and α-SecY antibodies bound to protein A–Sepharose beads.

immunoprecipitated by α-FtsY and α-SecY antibodies. This band probably reflects a crosslink between SecY and an N-terminally truncated FtsY derivative (FtsY*), which is routinely observed when cells are disrupted (Luirink *et al*, 1994).

We next tested whether an interaction between FtsY and SecY could also be shown by co-immunoprecipitations. After *in vitro* synthesis and integration of SecY at permissive temperatures, INV were isolated by ultracentrifugation, solubilized and subsequently immunoprecipitated with antibody–protein A beads. α-FtsY antibodies precipitated SecY in wt INV and to a lesser degree also in *secY40* INV (Fig 4A). This is probably due to impaired SecY integration into *secY40* INV even under permissive conditions. No radioactive material was immunoprecipitated by FtsY antibodies if SecE-depleted CM124 INV were used. In these INV, the cellular concentration of SecY is severely reduced, which precludes the integration of most membrane proteins (Koch & Müller, 2000). α-SecY antibodies precipitated a significant portion of the *in vitro*-synthesized SecY in wt INV and slightly less in *secY40* INV (Fig 4A). No immunoprecipitated material was observed in the absence of INV and only a weak band was observed in the presence of SecE-depleted CM124 INV. Similar results were observed if antibodies directed against YidC were used, which has been shown to be part of the SecY translocon (Scotti *et al*, 2000).

As the co-translational integration of SecY is SRP and FtsY dependent, we had to exclude the possibility that the interaction between FtsY and SecY—as observed by co-immunoprecipitations—is mediated through RNC complexes of SecY, which are bound to FtsY early during integration. This interaction should be sensitive to puromycin/EDTA treatment, which is routinely used to release ribosomes from nascent chain complexes. Fig 4A shows that this treatment did not diminish the amount of SecY immunoprecipitated with α-SecY or α-FtsY antibodies, which suggests that SecY integration intermediates do not contribute significantly to the observed SecY–FtsY interaction. This was further corroborated by performing immunoprecipitations with MtlA as the substrate. Like SecY, MtlA is co-translationally targeted to the Sec translocon by the SRP/FtsY pathway; thus, it interacts transiently with both SecY and FtsY before it is released into the lipid bilayer. If the above-described co-immunoprecipitations of FtsY with SecY reflect only these transient interactions during integration, α-FtsY antibodies should also immunoprecipitate MtlA. *In vitro*-synthesized MtlA, however, was significantly immunoprecipitated only with antibodies directed against MtlA but not with other antibodies (Fig 4A). This indicates that once completely integrated, an SRP/FtsY substrate such as MtlA does not remain in contact with the Sec translocon or FtsY. This suggests that SecY and FtsY not only interact because SecY is a substrate for the SRP/FtsY pathway, but also that membrane-integrated SecY provides a binding site for FtsY.

When co-immunoprecipitations were performed with *in vitro*-synthesized FtsY, α-SecY antibodies precipitated radiolabelled FtsY to almost the same degree as α-FtsY antibodies (Fig 4B). In the presence of *secY40* INV, slightly less FtsY was precipitated by α-SecY antibodies in comparison with wt INV, which may indicate that FtsY binds less efficiently to these mutant INV. When SecE-depleted CM124 INV were used, only α-FtsY antibodies were able to precipitate a small fraction of the membrane-bound FtsY; however, no FtsY was precipitated by α-SecY antibodies.

The Sec translocon co-purifies with FtsY

The presence of an FtsY–SecY complex was also confirmed by co-affinity purification methods. His-tagged FtsY was expressed *in vivo* and only the membrane-bound fraction of FtsY was purified by metal-affinity chromatography after solubilization with dodecyl-β-D-maltoside (DDM). In the eluted fraction containing purified FtsY, a 100 kDa protein corresponding to FtsY and a 75 kDa protein corresponding to FtsY* were visible after Coomassie blue staining (Fig 5, lane 3). Western blotting with anti-SecY and anti-YidC antibodies showed the presence of both translocon components in this fraction (Fig 5, lane 3). This was not due to unspecific binding of SecY or YidC to the metal affinity column, because when FtsY was expressed without His tag, neither SecY nor YidC was detected in the eluted fractions (Fig 5, lane 5). These data suggest that FtsY forms a rather stable complex with the SecY translocon.

It was shown recently that free FtsY shows only a low affinity for SRP and GTP (Shan & Walter, 2003), which led to the suggestion that a conformational change is required for high-affinity SRP–FtsY interaction. These data support previous mutagenesis studies, suggesting that a conformational change at the N-GTPase domain interface of FtsY is required for SRP binding (Lu *et al*, 2001). Both studies indicate that these conformational changes are induced through binding of FtsY either to membrane lipids or to a membrane receptor. Our data, showing a direct interaction between FtsY and SecY, suggest how these conformational changes are directly linked to the co-translational integration of bacterial membrane proteins. Binding of soluble FtsY to the membrane probably occurs initially by protein–lipid interaction

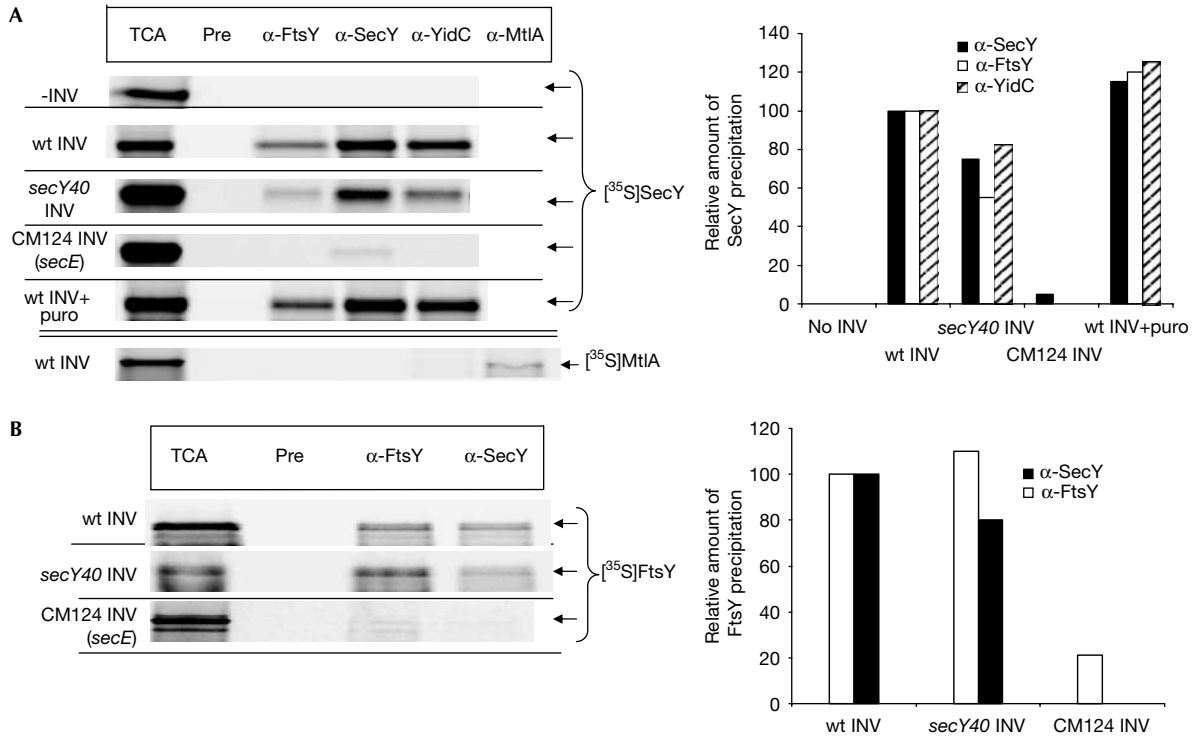

Fig 4 | SecY and FtsY are co-immunoprecipitated. (**A**) SecY and MtlA were synthesized *in vitro* at 37 °C for 30 min in the presence of wild-type (wt) or mutant INV. One aliquot of the reaction mixture was precipitated directly with trichloroacetic acid (TCA) and the remaining material was subjected to ultracentrifugation to collect the INV. Subsequently, the INV were solubilized with dodecyl-β-D-maltoside. As control, wt INV were treated with puromycin/EDTA (ethylenediamine tetra acetic acid) after integration of SecY and before solubilization (wt INV + puro). Immunoprecipitation was performed with protein A-coupled pre-immune serum (Pre) or antibodies, as indicated. Precipitated material was separated on SDS–PAGE and radioactively labelled bands were visualized and quantified using a PhosphorImager. The right-hand panel illustrates the quantification of the SecY data from the left-hand panel, and shows the percentage of precipitated SecY relative to the amount precipitated in wt INV, which was set to 100%. (**B**) FtsY was synthesized *in vitro* and treated as described in (**A**).

(Millman *et al*, 2001). Within the cell FtsY is in excess of SecY, therefore, lipid-associated FtsY probably represents the principal FtsY fraction. The initial binding of FtsY to lipids might favour a subsequent interaction of FtsY with the SecY translocon by trapping FtsY to the membrane. Lipid contact, however, is not sufficient to 'prime' FtsY for a functional interaction with the SRP–nascent chain complex, thereby preventing nascent chain release in the absence of an available translocon. A direct interaction of FtsY with the SecY translocon could induce the conformational change required to coordinate FtsY–SRP interaction with the subsequent dissociation of the signal anchor sequence from SRP. This mechanism would ensure that signal sequence release from SRP occurs only if a translocation channel is accessible. In the *secY40* mutant, this cycle is disturbed, either because the mutation reduces the affinity of SecY for FtsY, or because the mutated SecY is unable to induce the conformational change required for the subsequent FtsY–SRP interaction. Studies to discriminate between both possibilities are now in progress and will hopefully provide new insights on how the SecY translocon regulates the bacterial SRP cycle.

METHODS

Strains and plasmids. The strains and plasmids used for *in vitro* synthesis have been described previously (Kuhn & Wickner, 1985, Koch *et al*, 1999). In addition, the following strains were used: TY22 (*ompT*::*kan, secY40*; Taura *et al*, 1994) and BL21 Tuner™ (DE3) pLysS (Novagen, Bad Soden, Germany). The *Escherichia coli* strain TY22 DE3 pLysE, allowing T7-dependent expression of target genes, was constructed using the λDE3 lysogenization kit (Novagen). TY22 DE3 pLysE was transformed with pET19b-FtsY and expression of FtsY was induced by the addition of 0.2 mM isopropyl β-D-thiogalactopyranoside (IPTG). The expression level was analysed by Coomassie staining of SDS–polyacrylamide gel electrophoresis (SDS–PAGE) gels and western blot analyses.

***In vitro* synthesis and co-immunoprecipitations.** *In vitro* protein synthesis and the composition of the reconstituted transcription/translation system of *E. coli* have been described previously (Koch *et al*, 1999). Co-immunoprecipitations of *in vitro*-synthesized proteins were performed as follows: proteins were synthesized in the presence of INV for 30 min at 37 °C in a 200 µl reaction mixture. An aliquot was precipitated directly with trichloroacetic acid (TCA), and the remaining reaction mixture was subjected to centrifugation (55,000 r.p.m., 4 °C, for 30 min in a Beckman TLA100.3 rotor) to collect the INV. INV were resuspended in 100 µl lysis buffer (50 mM NaCl, 5 mM 6-aminohexanoic acid, 50 mM imidazole/HCl, pH 7) and DDM (Roche, Mannheim, Germany) was added to a final concentration of 2 mg/mg

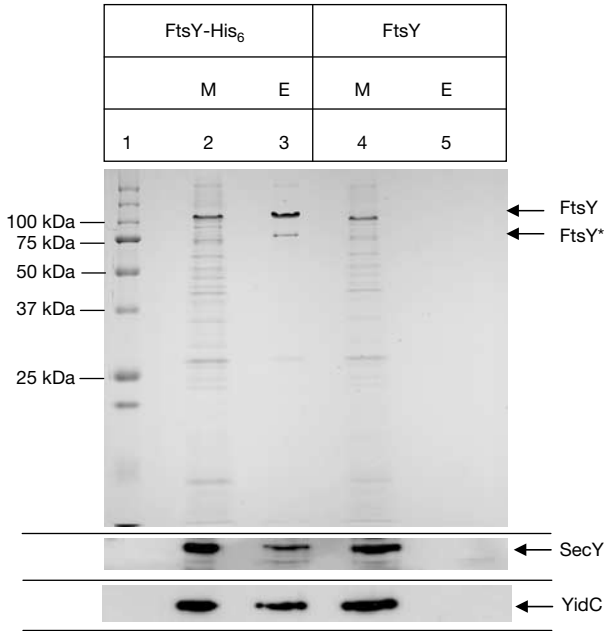

Fig 5 | The Sec translocon co-purifies with membrane-bound FtsY. FtsY with or without His_6 tag at the carboxyl terminus was purified from *Escherichia coli* crude membranes (M) by solubilization and metal-affinity chromatography. After purification, samples were separated on SDS–PAGE and stained with Coomassie blue. Eluted fractions containing purified His_6-tagged FtsY (E) were further analysed for the presence of SecY and YidC by western blot analyses. As a control, the procedure was repeated with untagged FtsY. FtsY* reflects an N-terminally truncated FtsY derivative, which is routinely observed in FtsY preparations (Luirink *et al*, 1994).

membrane proteins. After incubation for 10 min at 25 °C, solubilized proteins were isolated by centrifugation (45,000 r.p.m., 4 °C, for 30 min in a Beckmann TLA 45 rotor). The supernatant was incubated with antiserum coupled to protein A–Sepharose CL-4B (Amersham, Freiburg, Germany) in the presence of detergent buffer (0.5% DDM, 150 mM NaCl, 5 mM EDTA, 25 mM Tris–HCl, pH 6.8). After overnight incubation, the bound material was washed with detergent buffer, resuspended in 30 μl SDS loading buffer lacking DTT and separated on 13% SDS–PAGE. Puromycin treatment of INV was performed after *in vitro* synthesis and integration but before solubilization. Isolated INV were incubated with 0.1 M puromycin and 0.02 M EDTA for 15 min at 37 °C. INV were isolated by centrifugation and solubilized as described above. For details on chemical cross-linking and affinity purification, see the supplementary information online.

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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